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Original Article

In vivo studies could not confirm *in vitro* prophylactic synergism between *Moringa* essential oil and *Lactobacillus reuteri* (MT180537)

Estudos *in vivo* não puderam confirmar o sinergismo profilático *in vitro* entre o óleo essencial de *Moringa* e *Lactobacillus reuteri* (MT180537)

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Abstract

Aerobic vaginitis (AV) is a recently defined vaginal recurring infection, which is treated with antibiotics. However, excessive and prolonged use of antibiotics disrupts healthy vaginal microflora and leads to the emergence of antibiotic resistance among pathogens. This situation has directed researchers to explore alternative antimicrobials. The current study describes *in vitro* and *in vivo* antimicrobial efficacy and pharmaceutical interactions between plant essential oils (EOs) and five lactic acid bacteria (LABs), isolated from the healthy vagina, against *E. faecalis,* one of the major etiological agents of AV. *In vitro* experiments confirm good antimicrobial activity of both plant EOs and cell free supernatant (CFS) from LABs. Based on high antimicrobial efficacy, *Moringa* essential oil (MO) was selected to determine its nature of interaction with CFS of five LAB strains. Synergism was recorded between MO and CFS of *L. reuteri* (MT180537). To validate *in vitro* findings, prophylactic responses of individual and synergistic application of MO and *L. reuteri* (MT180537) were evaluated in an *E. faecalis* (MW051601) induced AV murine model. The prophylactic efficacy was evidenced by a reduction in intensity of clinical symptoms, *E. faecalis* (MW051601) count per vaginal tissue along with a reduction in AV associated changes in histological markers of infection in animals receiving *Moringa* essential oil and *L. reuteri* (MT180537) alone or in combination. However, significant synergism between *Moringa* essential oil and *L. reuteri* (MT180537) could not be observed. Our data confirms the importance of *in vivo* experiments in deducing pharmacological interactions.

Keywords: aerobic vaginitis, essential oil, lactic acid bacteria, murine model, synergistic interaction.

Resumo

Vaginite aeróbica (VA) é uma infecção vaginal recorrente definida recentemente, que é tratada com antibióticos. No entanto, o uso excessivo e prolongado de antibióticos perturba a microflora vaginal saudável e leva ao surgimento de resistência aos antibióticos entre os patógenos. Esta situação levou os pesquisadores a explorar antimicrobianos alternativos. O presente estudo descreve a eficácia antimicrobiana *in vitro* e *in vivo* e as interações farmacêuticas entre óleos essenciais vegetais (OE) e cinco bactérias lácticas (BAL), isoladas de vagina sã, contra *E. faecalis*, um dos principais agentes etiológicos da AV. Os experimentos *in vitro* confirmam a boa atividade antimicrobiana de ambos os EOs de plantas e sobrenadante livre de células (CFS) de LABs. Com base na alta eficácia antimicrobiana, o óleo essencial de *Moringa* (MO) foi selecionado para determinar sua natureza de interação com o sobrenadante livre de células (CFS) de cinco cepas de LAB. Sinergismo foi registrado entre MO e CFS de *L. reuteri* (MT180537). Para validar os resultados *in vitro*, as respostas profiláticas da aplicação individual e sinérgica de MO e *L. reuteri* (MT180537) foram avaliadas em um modelo murino AV induzido por *E. faecalis* (MW051601). A eficácia profilática foi evidenciada por uma redução na intensidade dos sintomas clínicos, contagem de *E. faecalis* (MW051601) por tecido vaginal, juntamente com uma redução nas alterações associadas a AV nos marcadores histológicos de infecção em animais que receberam óleo essencial de *Moringa* e *L. reuteri* (MT180537) sozinho ou em combinação. No entanto, não foi possível observar sinergismo significativo entre o óleo essencial de *Moringa* e *L. reuteri* (MT180537). Nossos dados confirmam a importância dos experimentos *in vivo* na dedução de interações farmacológicas.

Palavras-chave: vaginite aeróbia, óleo essencial, bactéria láctica, modelo murino, interação sinérgica.

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1. Introduction

Vaginitis is an inflammation of the vagina that predominantly affects reproductive aged women (Jeng et al., 2020). It is generally characterized by vaginal dysbiosis and is associated with an increased risk of preterm birth and pelvic inflammatory infections. The most common dysbiosis of the vaginal microbiome are vulvovaginal candidiasis (VVC), trichomoniasis, bacterial vaginosis (BV) and aerobic vaginitis (AV) (van de Wijgert and Jespers, 2017). Among these, AV is a newly defined vaginal infection, which is caused by aerobic pathogens such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and group B *Streptococcus* spp. (Tempera et al., 2004). *Enterococcus* species were neglected as pathogens to humans for many years and believed to be insignificant medically (Moreno et al., 2006). Recently, *enterococci* have turned out to be one of the most imperative nosocomial pathogens, causing mortality up to 61% (Lopes et al., 2005). *E. faecalis* is considered the etiological agent of almost 80 percent of human infections and is incriminated in the pathogenesis of AV at a rate of 32.26% followed by *E. coli* (25.8%) (Sangeetha et al., 2015; Daood et al., 2020). The symptoms of AV include thinned vaginal mucosa, increased vaginal inflammation, and invariably an abundant yellowish foul-odor vaginal discharge (Donders et al., 2009). Increased maternal age, multiple sex partners, previous spontaneous abortions and altered vaginal microbial flora including depressed vaginal *Lactobacillus* spp. are considered predisposing factors, which support pathogen colonization (Rampersaud et al., 2012).

Currently, there is no widely accepted standard approach for the treatment of AV except antibiotics. However, drug resistance among pathogens has resulted in treatment failures and frequent recurrent vaginal infections (Ben-Ami, 2018; Abd Ellah et al., 2019). This situation has directed researchers to explore efficient alternative antimicrobials, preferably from natural sources.

Plant essential oils (EOs) have long been recognized for their antiviral, antifungal, antibacterial, antioxidant and insecticidal properties (Wang et al., 2018; Ma and Yao, 2020). Similarly, probiotics are recognized as live microorganisms that confer health benefits on the host when administered in adequate amounts (FAO, 2002). Probiotic lactobacilli are prompted to prevent the growth of pathogens by competitive exclusion and/or production of antimicrobial secondary metabolites (lactic acid, hydrogen peroxide and bacteriocins) (Tempera et al., 2004; Bassolé and Juliani, 2012).

Previous studies have also suggested a potential synergy between plant EOs and other antimicrobial agents against multi-drug resistant pathogens (van Vuuren et al., 2009; Rakholiya and Chanda, 2012). These studies were based on the common assumption that essential oils can be used for their biological activities as well as synergistic enhancers in pharmaceutical formulations (Kamatou et al., 2006). The objective of the current work was to investigate the individual and synergistic antimicrobial efficacy of plant EOs and lactic acid bacteria against *E. faecalis* by employing both *in vitro* and in *vivo* approaches.

2. Materials and Methods

2.1. Essential oils

Naturally originated commercial essential oils of four medicinal plants *Moringa oleifera* Lam. (Drumstick), *Colchicum luteum* Baker. (Suranjan), *Celastrus paniculatus* Willd. (Black oil) and *Sesamum indicum* L. (Sesame) were purchased from the local market in Lahore, Pakistan. Stock solutions of each EO were prepared in DMSO (Dimethyl sulfoxide) and diluted to obtain a final concentration of 2% (v/v). Each EO was also buffered to pH 7.0 and to enhance solubility of EOs, Tween-80 (Sigma-Aldrich) was added at the final concentration of 0.001%. Moxifloxacin was used as a reference drug.

2.2. Lactic acid bacteria (source and characteristics)

Five strains of LABs were originally isolated from vaginal swabs of healthy women by professional clinicians. The strains were cultured on De Man, Rogosa and Sharpe (MRS) for 24-48 h at 37 °C under anaerobic conditions. The strains were screened morphologically and biochemically (Bergey et al., 1994). The strains were identified as *Lactobacillus reuteri* (MT180537), *Pediococcus pentosaceus* (MT176555), *Lactobacillus pontis* (MW362838), *Lactobacillus brevis* (MW051029) and *Lactobacillus brevis* (MW362790) based on 16S rRNA gene sequencing and accession numbers were obtained from NCBI (National Center for Biotechnology Information). *L. acidophilus* ATCC 4356 was included as a reference strain in *in vitro* antimicrobial assays. LABs were confirmed for their probiotic characteristics based on acid, bile salt, NaCl, lysozyme tolerance, self-aggregation, co-aggregation and good adherence ability (Data not shown).

2.3. Pathogen and growth conditions

E. faecalis (MW051601) was initially obtained from patients of AV. Pure culture was stored at -20 °C in Brain Heart Infusion broth (BHI) containing 25% (v/v) glycerol. Before each experiment, the strain was inoculated on a BHI agar plate to ensure purity and optimal growth.

2.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC values of *Moringa* essential oil and CFS from LABs were evaluated using microdilution assay against *E. faecalis* (MW051601) (CLSI, 2017). Briefly, 50 µL of serial two-fold dilutions of both MO (25-0.05 mg mL^{-1}) and CFS corresponding to 3.8-0.01 mg mL^{-1} were prepared by incorporating into Mueller-Hinton broth (MHB). Then, 50 µL of *E. faecalis* (MW051601) suspension containing $5x10^5$ CFU mL^{-1} was added resulting in a final volume of 100 µL in each well. The last well, in each row, containing equal ratio of MHB and test strain without any antimicrobial was used as a positive control. For negative control, 50 µL of each antimicrobial at highest concentration was added to 50 µL of MHB. The plates were incubated at 37 °C for 24 h under aerobic conditions. The survival of *E. faecalis* (MW051601) was evidenced by appearance of red color following addition of 20 µL/well of 2,3,5-triphenyl

tetrazolium chloride (TTC) and incubation at 37 °C for 30 min. The lower antimicrobials that restricted visible growth were termed the MIC while the lowest concentration of antimicrobial that kills 99.9% of the indicator strain was termed MBC. The experiments were performed in triplicate.

2.5. Determination of the Fractional Inhibitory Concentration (FIC) index

The interaction between *Moringa* essential oil and CFS from LABs against *E. faecalis* (MW051601) was determined using checkerboard assay following van Vuuren et al. (2009). In brief, 50 µL of serially two-fold diluted antimicrobials were dispensed to the wells of 96-well microtiter plate in a horizontal (*Moringa* essential oil, 25-0.05 mg mL^{-1}) and vertical direction (CFS, 3.8-0.01 mg mL^{-1}) thus varying the concentrations of each antimicrobial along matrix. *E. faecalis* (MW051601) ($5x10^5$ CFU mL^{-1}) was inoculated in each well except in row H. Wells (column 12) containing pathogen alone in absence of any antimicrobial were used as a positive control while wells (row H) with each antimicrobial alone were used as an additional media sterility control. After incubation at 37 °C overnight, results were interpreted as mentioned for MIC assay. The FIC index of interaction between *Moringa* essential oil (drug X) and CFS (drug Y) was calculated using the following Equation 1:

$$FIC\ index = FICX + FICY = \frac{\left[MIC\ (X\ in\ presence\ of\ Y) / MIC\ (X\ alone)\right]}{\left[MIC\ (Y\ in\ presence\ of\ X) / MIC\ (Y\ alone)\right]} + \quad (1)$$

FICX is the MIC of *Moringa* essential oil alone, FICY is the MIC of CFS alone. Where X is the concentration of *Moringa* essential oil in a well that is MIC in its row, Y is the concentration of CFS in a well that is the MIC in its column. The nature of interaction was considered as synergistic (FIC ≤ 0.5), additive (0.5 < FIC ≤ 1), indifference if value is 1 < FIC ≤ 4 and antagonism at FIC > 4.0 (Hübsch et al., 2014).

2.6. Experimental animals

Two-month old healthy female albino mice (n=50) weighing from 20 to 30 g were obtained from inbred stocks of Government College University (GCU) Lahore, Pakistan. Mice were housed in steel cages under standard conditions (12h light/dark period, 25-27 °C temperature and 45-60% humidity). Vital grower feed and fresh water were provided *ad libitum* and animals were acclimatized for one week prior to the commencement of the study.

2.7. Media for selective re-isolation of pathogen

Antibiotic susceptibility profile of mice flora and *E. faecalis* (MW051601) was performed initially to select the antibiotic/s to which mice flora was sensitive but *E. faecalis* (MW051601) was resistant. The MIC of selected antibiotic (Clindamycin) was performed using broth microdilution assay and antibiotic equivalent to 0.1 MIC was added in Eosin Methylene Blue (EMB) agar. The modified media (supplemented with Clindamycin) was confirmed for selective re-isolation of *E. faecalis* (MW051601), restricting the growth of indigenous enterococcus.

2.8. Inoculum preparation

Five serial 10-fold dilutions of suspensions (OD_{600nm}=1.00 ± 0.02) of both *L. reuteri* (MT180537) and *E. faecalis* (MW051601) were prepared in sterile physiological saline. The 50 µL of different dilutions of *L. reuteri* (MT180537) and *E. faecalis* (MW051601) were spread on the surface of MRS and EMB agar plates respectively. The plates were incubated at 37 °C overnight and viable cells were counted by following Formula 2:

$$CFU / mL = \left(\frac{number\ of\ colonies /}{volume\ of\ culture\ spread\ on\ plate}\right) \times Dilution\ factor \quad (2)$$

The inoculum sizes of ~ 1×10^8 and 5×10^5 of *L. reuteri* (MT180537) and *E. faecalis* (MW051601) respectively (each per 50 µL of physiological saline) were used for inoculation in mice (De Gregorio et al., 2014).

2.9. Experimental procedures

The schedule of treatments is given in Figure 1. Total fifty mice were randomized into five experimental groups (10 mice/group). Animals were immunocompromised with subcutaneous exposure to β-estradiol valerate at the concentration of 0.8 mg/kg/week. Mice in groups I-III were intravaginally (i.vag.) treated with 50 µL of *Moringa* essential oil (12.50 mg mL^{-1}), *L. reuteri* (MT180537) (1×10^8 CFU) and mixture (1×10^8 CFU mixed in 12.50 mg mL^{-1} of *Moringa* essential oil) respectively twice a day for two consecutive days. Later on, mice of group I-IV were challenged with vaginal perfusion of 50 µL of *E. faecalis* (MW051601) (5×10^5 CFU) once daily for two days. Mice in Group V were treated similarly with physiological saline.

2.10. Measurement of clinical signs

During the course of the study, animals were monitored for the severity of the AV using the clinical index, which consisted of five parameters *viz*., discharge turbidity, redness, inflammation, animal behavior and loss of fur around vagina. All symptoms were given equal weightage. Animals were scored (0-1) depending on presence or absence of symptom. After scoring, cumulative score of clinical symptoms was used in statistical analysis for comparison of clinical index among groups.

2.11. Sampling of vaginal tissue

On 12^{th} dpi, animals were sacrificed under a high dose of ketamine (200 mg kg^{-1}). Vaginal tissues were harvested, half part of vaginal tissue was weighed to determine bacterial load and the rest of the half part was fixed in neutral buffered-formalin (10%) for histopathological studies.

2.12. Bacterial load per vaginal tissue

The *E. faecalis* (MW051601) count in vaginal tissue was measured by homogenizing weighted tissue in 2 mL sterile saline. Different 10-fold dilutions of the homogenate (0.1 mL) were plated on clindamycin supplemented EMB and CHROM agar following incubation for 24 h at 37 °C. While indigenous enterococci are detected on CHROM agar, no mice were colonized with strains that resemble

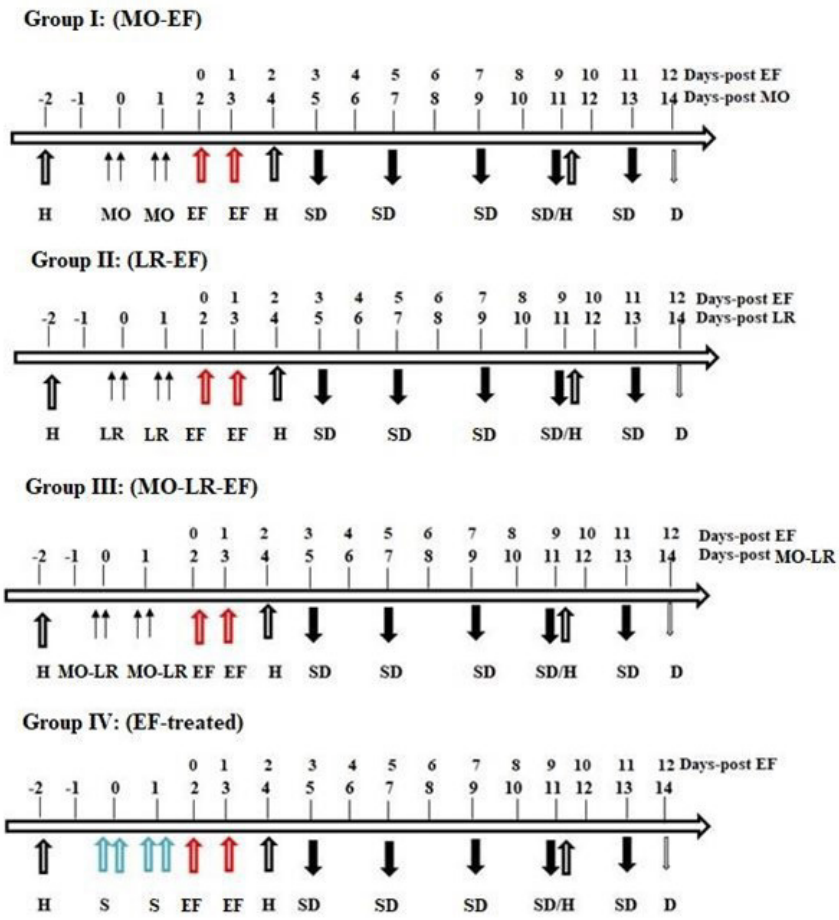

Figure 1. Experimental design, schedule of treatments and sampling days. H: β-Estradiol 17 valerate (0.8 mg/kg/week). The arrows illustrate i. vag. inoculations of: Saline (S), *E. faecalis* (MW051601) (EF=5×10^5 CFU), *Moringa* essential oil (MO=12.50 mg mL^{-1}), *L. reuteri* (MT180537) (LR=1×10^8 CFU) and MO-LR mixture corresponding to 1×10^8 CFU of *L. reuteri* (MT180537) mixed in 12.50 mg mL^{-1} of *Moringa* essential oil) Sampling day (SD), Dissection (D).

E. faecalis (MW051601), as no colonies appeared on EMB agar supplemented with clindamycin that selects for *E. faecalis* (MW051601).

2.13. Histopathology

The vaginal tissues fixed in 10% neutral buffered formalin, were processed for histopathological examination. Thin vaginal sections (5 µm) stained with hematoxylin-eosin were observed and images were captured using camera fitted light microscope (Labomed, USA). The transitional epithelial thickness and exfoliation were measured following Gilbert et al. (2013) with slight modifications.

2.14. Statistical analysis

Data were expressed as mean ± standard error and statistical analysis was performed in GraphPad Prism software (version 5.0.0). Non parametric data were analyzed by Kruskel Wallis H-test followed by Mann-Whitney U test for pairwise comparison while parametric data were analyzed with one-way Analysis of Variance (ANOVA) followed by Tukey's test. Statistical significance was considered at p value < 0.05.

3. Results

3.1. Microdilution assay

Plant EOs and CFS from LABs produced variable MIC and MBC values as shown in Table 1. MO demonstrated MIC and MBC values of 12.5 mg mL^{-1} and 25 mg mL^{-1} respectively against *E. faecalis* (MW051601). Among LABs, MIC value ranged from 1.90 to 0.48 mg mL^{-1} and MBC from 3.80 to 0.95 mg mL^{-1} respectively. *P. pentosaceus* (MT176555) showed higher antimicrobial activity, with MIC and MBC values of 0.48 and 0.95 mg mL^{-1} respectively.

3.2. Determination of the Fractional Inhibitory Concentration (FIC) index

The data of the FIC index was expressed in terms of interaction between MO and CFS of LABs that is summarized in Table 2. Marked synergistic effect (FIC index = 0.25) was observed between MO with CFS of *L. reuteri* (MT180537). LABs including *L. pontis* (MW362838), *L. brevis* (MW051029), *L. brevis* (MW362790) and *L. acidophilus* ATCC 4356 (reference strain) showed indifferent outcomes. However, *P. pentosaceus* (MT176555) was antagonistic when combined with MO.

3.3. Clinical symptoms

Mice exposed to *E. faecalis* (MW051601) (positive control) presented clinical signs (vaginal discharge turbidity, redness, inflammation, animal irritation and thinning of fur around vagina) when compared with the negative control. Animals treated with *Moringa* essential oil, *L. reuteri* (MT180537) or MO-LR (*L. reuteri*-MT180537 mixed in *Moringa* essential oil) showed a significantly lower clinical index as compared to positive control group ($p < 0.05$). Regarding the parameters of clinical index, few conditions (redness and discharge) initially appeared in all prophylactic groups that reduced with the passage of time. In addition, animals treated with mixture did not show any of the symptoms by 11 dpi (Table 3).

3.4. Tissue bacterial load

Bacterial load of *E. faecalis* (MW051601) was 5.58 ± 0.27 CFU g^{-1} in positive control (group IV) while significantly lower count (1.81 ± 0.68 CFU g^{-1}) was observed in *L. reuteri* (MT180537) treated group (group II) with reference to positive control (group IV). Moreover, lowest *E. faecalis* (MW051601) count (1.17 ± 0.29) was detected in the group treated with the MO-LR mixture prior to *E. faecalis*-MW051601 exposure (group III) (Figure 2).

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in mg mL^{-1} of selected plant essential oils and cell free supernatant from lactic acid bacteria against *E. faecalis* (MW051601).

Antimicrobial	*E. faecalis* (MW051601)	
	MIC*	MBC#
Essential oil		
Moringa oleifera	12.5	25
Celastrus paniculatus	25	50
Sesamum indicum	25	50
Colchicum luteum	25	50
Lactic acid bacteria		
L. reuteri (MT180537)	0.95	1.90
P.pentasaceus (MT176555)	0.48	0.95
L. pontis (MW362838)	0.95	1.90
L. brevis (MW051029)	0.95	1.90
L. brevis (MW362790)	1.90	3.80

*Minimum inhibitory concentration; #Minimum bactericidal concentration.

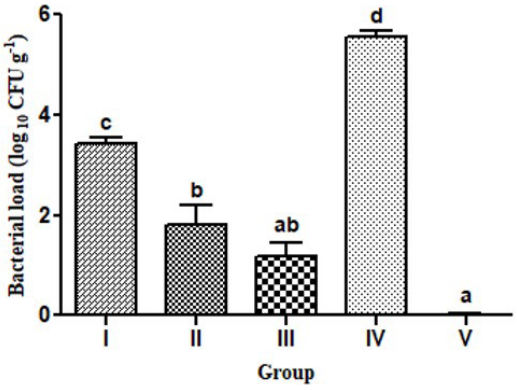

Figure 2. Bacterial load (CFU g^{-1} of vaginal tissue) at 11-day post infection. Data were analyzed using a one-way Analysis of Variance (ANOVA) followed by Tukey's test. The results are expressed as Mean ± SEM. Different letters on bars show significant differences at $p < 0.05$. I= *Moringa* essential oil, II= *L. reuteri* (MT180537), III= MO-LR mixture, IV= *E. faecalis* (MW051601), V= Negative control.

Table 2. Determination of Fractional Inhibitory Concentration (FIC) index (mg mL^{-1}) between *Moringa* essential oils (MO) and Cell free Supernatant (CFS) from lactic acid bacteria against *E. faecalis* (MW051601).

Antimicrobial Components	MICX* (Alone)	MICY† (Alone)	MIC‡ (mixed)	MIC§ (mixed)	FIC index	Outcome
L. reuteri & MO	12.5	0.95	1.56	0.12	0.25	Synergy
P. pentosaceus & MO	12.5	0.48	12.5	1.90	4.96	Antagonism
L. pontis & MO	12.5	0.95	25	0.24	2.25	Indifference
L. bravis & MO	12.5	0.95	25	0.95	3.00	Indifference
L. brevis & MO	12.5	1.90	25	1.90	3.00	Indifference
L. acidophilus & MO	12.5	1.90	12.5	0.48	1.25	Indifference

*X is the concentration (mg/mL) of MO in well that is MIC in its row; †Y is the concentration of active ingredient in CFS (mg/mL) in well that is the MIC in its column; ‡MIC (mg/mL) of MO in presence of CFS; §MIC (mg/mL) of CFS in presence of MO. Based on FIC value, the nature of interaction was considered as synergistic (FIC ≤ 0.5), additive (0.5 < FIC ≤ 1), indifferent (1 < FIC ≤ 4) or antagonism (FIC > 4.0).

Table 3. Cumulative score of clinical symptoms in animals.

Day post infection	Group	Cumulative score	Mean Rank	Kruskal Wallis H (p<0.05)
Day 3	I	1	26	0.900
	II	1	26	
	III	1	26	
	IV	1	26	
	V	0	23.5	
Day 5	I	3	21.4	0.000
	II	3	22.65	
	III	3	22.65	
	IV	22	44.3	
	V	0	16.5	
Day 7	I	6	24.45	0.001
	II	7	27.5	
	III	6	23.4	
	IV	24	38.65	
	V	0	13.5	
Day 9	I	10	28.15	0.000
	II	7	25.45	
	III	4	21.7	
	IV	33	39.7	
	V	0	12.5	
Day 11	I	10	32.1	0.001
	II	6	25.5	
	III	0	15	
	IV	31	39.9	
	V	0	15	

Data were analyzed using Kruskel Wallis H-test followed by Mann-Whitney U test for pairwise comparison. Results were considered significant at $p < 0.05$. Mice in groups I-III were (i.vag.) treated with 50 µL of essential oil (12.50 mg mL^{-1}), *L. reuteri* (MT180537) (1×10^8 CFU) and mixture (1×10^8 CFU mixed in 12.50 mg mL^{-1} of *Moringa* essential oil) respectively twice a day for two consecutive days. Later on, mice of group I-IV were challenged with vaginal perfusion of 50 µL of *E. faecalis* (MW051601) (5×10^5 CFU) once daily for two days. Mice in Group V were treated similarly with physiological saline.

3.5. Histopathological examination

E. faecalis (MW051601) treated mice (positive control) presented significant damage in mucosal tissue that was evident by increased thickness of epithelium and high epithelial cell exfoliation (Figure 3). Mice in *Moringa* essential oil, *L. reuteri*-MT180537 and MO-LR treated groups were found resistant to mucosal tissue damage that was evident by less degree of changes in epithelium similar to negative control (Figure 4).

4. Discussion

For the last four decades, antibiotics have been used for the treatment of urogenital infections. However, antibiotic therapy, especially when prolonged, leads to side effects *viz*., neutropenia, peripheral neuropathy and pancreatitis (Ferreira et al., 2011). Among natural products, plant EOs and probiotics have been investigated for their antiviral, antifungal and antibacterial properties by various authors (Kaur and Tiwari, 2016; Iseppi et al., 2020) but their synergistic antibacterial ability has not been reported so far. In the current study, *in vitro* and *in vivo* pharmaceutical interactions between four plant EOs and five strains of LABs were evaluated against *E. faecalis*, which is considered one of the most frequently isolated pathogens from AV cases.

Plant EOs are generally used as a promising alternative remedy for topical bacterial infections (Bogavac et al., 2017). The selection of essential oils was based both on ethnomedicinal use and on the proven antibacterial and/or antifungal activity of these oils. Several studies have reported an inverse relationship between the number of *Lactobacillus* spp. and pathogens in the female reproductive tract (Kamińska and Gajecka, 2017). *Lactobacillus* spp.

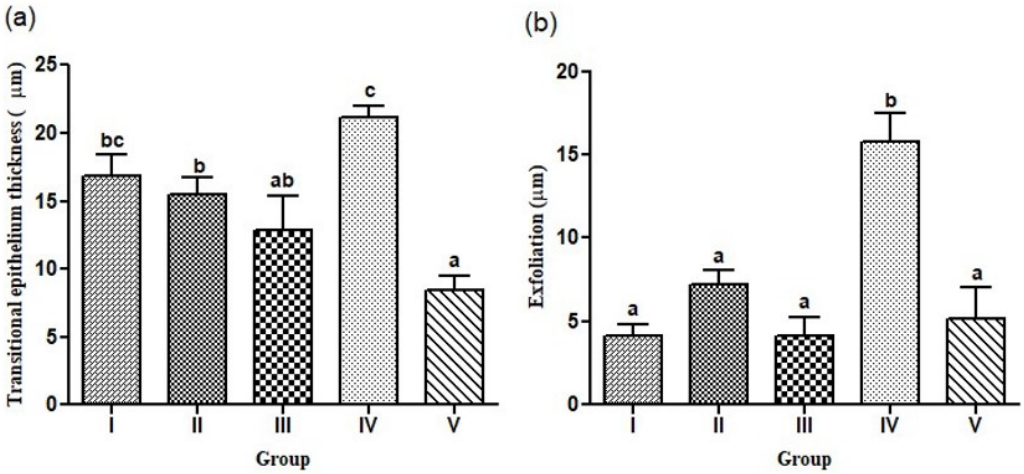

Figure 3. Influence of prophylactic application of *Moringa* essential oil, *L. reuteri* (MT180537) and MO-LR mixture on vaginal histopathological changes; (a) epithelial thickness, (b) exfoliation in AV induced mice. Data were analyzed using a one-way Analysis of Variance (ANOVA) followed by Tukey's test. The results are expressed as Mean ± SEM. Different letters on bars show significant differences at $p < 0.05$. I= *Moringa* essential oil, II= *L. reuteri* (MT180537), III= MO-LR mixture, IV= *E. faecalis* (MW051601), V= Negative control.

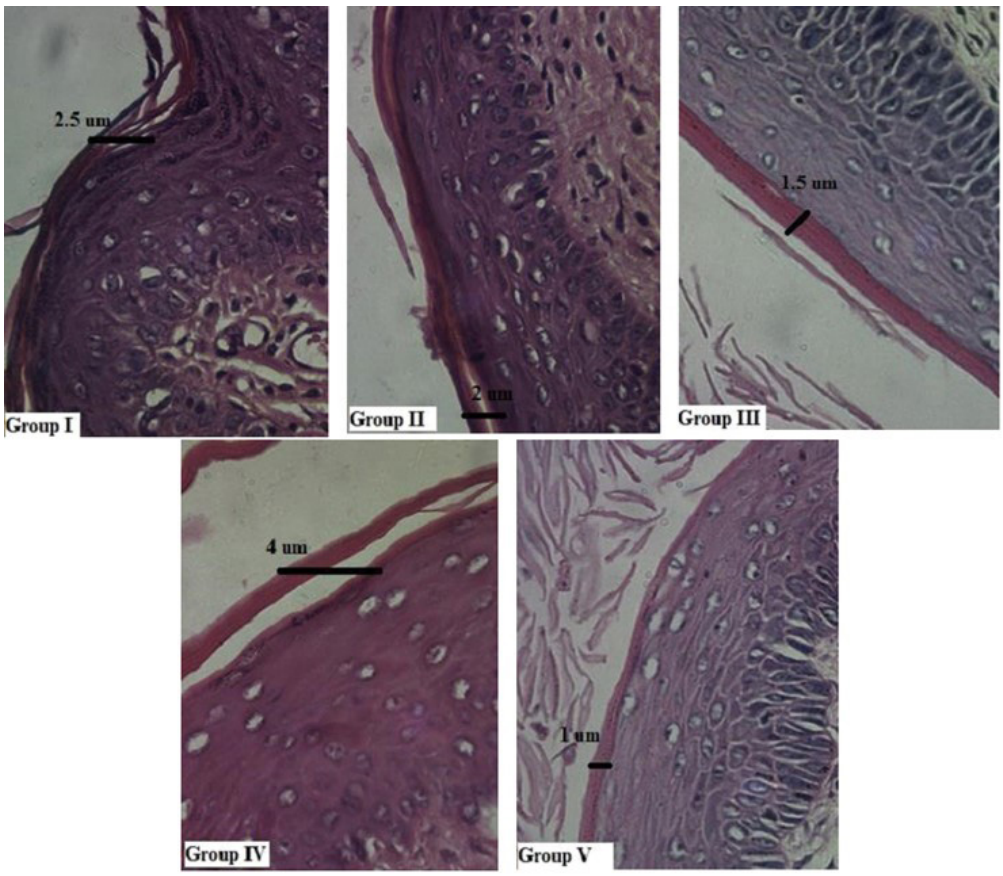

Figure 4. Influence of prophylactic application of MO, *L. reuteri* (MT180537) and MO-LR mixture on *E. faecalis* (MW051601) induced histopathological changes in vaginal transitional epithelium. Mice in groups I-III were (i.vag.) treated with 50 µL of *Moringa* essential oil (12.50 mg mL^{-1}), *L. reuteri* (MT180537) (1×10^8 CFU) and mixture (1×10^8 CFU mixed in 12.50 mg mL^{-1} of *Moringa* essential oil) respectively twice a day for two consecutive days. Later on, mice of group I-IV were challenged with vaginal perfusion of 50 µL of *E. faecalis* (MW051601) (5×10^5 CFU) once daily for two days. Mice in Group V were treated similarly with physiological saline. All photomicrographs were captured at 100X magnification.

are known to provide protection against pathogens through their secondary metabolites (lactic acid, hydrogen peroxide and bacteriocins) (Tempera et al., 2004; Bassolé and Juliani, 2012).

In the current study, the antimicrobial activity of EOs and CFS from LABs was determined by microdilution assays while their interaction was evaluated using a checkerboard assay. The results indicated that MO has superior antimicrobial activity among EOs. Virk et al. (2019) has reported antagonistic activity of MO against *S. aureus* and *E. coli*. LABs showed MIC values ranging from 1.90 to 0.48 mg mL^{-1} against *E. faecalis* (MW051601) which is in accordance with Nazareth et al. (2019), who reported MIC values of LABs ranging from 125 to 4 g/L against fungi. Based on the higher antimicrobial efficiency of MO, further experiments were performed to evaluate the antimicrobial activity of MO, *L. reuteri* (MT180537) and MO-LR against *E. faecalis* (MW051601).

The synergistic interaction in combination therapies is believed to reduce the required concentration of the active molecules in each component and enhance their range of action, thereby decreasing the possible side effects, often related to monotherapy regimens (Walkenhorst, 2016). In the presence of MO, the antimicrobial potential of CFS from *L. reuteri* (MT180537) increased (FIC index = 0.25), suggesting marked synergism between them. In spite of the fact that the MIC of *P. pentosaceus* (MT176555) was lower than the MIC of the other *Lactobacillus* spp., it interacts negatively with MO resulting in an antagonistic outcome in checkerboard assay. The results indicated that the antibacterial potential does not depend only on the presence of secondary metabolites but also on the amount and type of interactions with their constituents. Dzotam et al. (2015) found synergistic effect among three plants (*Xanthosoma mafaffa* Lam., *Moringa oleifera* L.Schott and *Passiflora edulis* Sims.) against multi-drug resistant Gram negative bacteria (FIC index < 0.5). In another study, Aminnezhad et al. (2015) reported FIC indices (0.124 and 0.56) of CFS from *L. rhamnosus* and aminoglycoside antibiotics (Gentamicin and Amikacin) while FIC indices from *L. casei* with the same antibiotics were 0.124 and 0.312, respectively. Based on the FIC index, *L. reuteri* (MT180537) was selected as promising candidate to determine *in vivo* synergistic efficacy using an *E. faecalis* (MW051601) induced AV murine model. Additionally, *L. reuteri* (MT180537) rather than CFS was preferred due to the fact that *L. reuteri* (MT180537) will act as a continuous source of secondary metabolites in the murine model.

The murine model is an assessment tool for research outcomes of infections and treatments. AV murine model was based on the subcutaneous administration of β-estradiol valerate due to its role in colonization of pathogens in mice through induction of the pseudo estrus stage (Calderon et al., 2003). Obvious, *E. faecalis* (MW051601) count emerged in the vaginal wash 11 days post infection at 0.8 mg/kg/week.

Previously, the link between clinical signs and pathogenesis of *E. faecalis* (MW051601) in the reproductive tract of females was not fully known. In this study, the first evidence of prophylactic effects of MO, *L. reuteri* (MT180537) and MO-LR was noticed by a decrease in the number of clinical symptoms (discharge turbidity, redness, inflammation, animal behavior and loss of fur around the vagina) in MO, *L. reuteri* (MT180537) and MO-LR treated mice as compared to untreated mice (negative control). The clinical symptoms improved in MO and *L. reuteri* (MT180537) treated groups but completely vanished in MO-LR treated animals. In a similar study, Zhou et al. (2019) recorded vaginal redness, swelling and turbidity of discharge as clinical parameters in the BV model and reported significant improvement in clinical symptoms following *C. butyricum*, another probiotic strain.

The growth inhibition of pathogens (lower bacterial load) is considered a gold standard for effective treatment outcome. In this study, colonization was determined in terms of *E. faecalis* (MW051601) count per vaginal tissue. The data further provided evidence in favor of prophylactic efficacy of three formulations and displayed significantly lower *E. faecalis* (MW051601) load as compared to positive control. The results indicated that prophylactic efficacy of lactobacilli may involve the blockage of receptor sites by steric hindrance, competition for receptors and production of secondary metabolites.

Consistent with other parameters, histopathological analysis also revealed the prophylactic efficacy of *Moringa* essential oil, *L. reuteri* (MT180537) and MO-LR that was evidenced by a lower degree of vaginal epithelial damage, decreased exfoliation and thickness on 12^{th} dpi in prophylactic groups. Gilbert et al. (2013) reported that *Gardnerella vaginalis* (GV) induced BV caused abnormal pathological changes in the vaginal tissues of mice.

5. Conclusion

In culmination, plant essential oils and LABs show antagonistic potential against *E. faecalis* (MW051601). *Moringa* EO boosts antagonistic hallmarks of *L. reuteri* (MT180537) in *in vitro* findings. Moreover, prophylactic effects were observed in case of three formulations applied as a topical preventive strategy but no significant synergism was observed in the murine model employed in this study.

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